Cell Communication, Stochastic Cell Responses, and Anthocyanin Pattern in Mustard Cotyledons

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The role of intercellular signals in plant development was investigated using phytochrome-induced formation of anthocyanin in cotyledons of white mustard as a model system. The problem was approached by irradiating different subregions of the cotyledon with a microbeam. This technique was combined with in situ hybridization of chalcone synthase mRNA after irradiation of the entire cotyledon. Individual cells that exhibited all-or-none responses with a resultant stochastic, patchy pattern were examined during early stages of anthocyanin synthesis. It was demonstrated that the responses of individual cells were subsequently integrated by long-range inhibitory signals. This process led to ordered and gradually developing patterns that could be detected when final stages were analyzed at the whole-organ level. The significance of these findings is discussed in terms of efforts toward a general understanding of photomorphogenesis in plants.

INTRODUCTION

Coordination of the responses of individual cells is a prerequisite for plant development. Such coordination might stem from precise genetic programs that predetermine exact fates of individual cells. Alternatively, individual cells might "choose" between several developmental pathways in response to putative signals provided by their neighbors. One of the most dramatic switches in development of plants is the transition from dark development (skotomorphogenesis) to photomorphogenesis (Mohr, 1972). We used this developmental switch as a model to determine whether such intercellular signals exist and to examine the way in which they might affect the photomorphogenetic responses of individual cells. This problem was addressed by analyzing aspects of the formation of anthocyanin in the cotyledons of white mustard.

In white mustard, the light-quenching flavonoid anthocyanin accumulates in the vacuoles of the cells in the lower epidermis of the cotyledon upon illumination (Mohr, 1966, 1972). The response is induced by phytochrome and has a characteristic spatial and temporal pattern: formation of anthocyanin is initiated at margins and veins, and then it gradually becomes apparent in the intercostal regions (Steinitz and Bergfeld, 1977). The timing of this response is independent of environmental factors (Steinitz et al., 1976). We chose to study this response because the synthesis of anthocyanin in mustard cotyledons is one of the best-investigated models of photomorphogenic pattern formation (Mohr, 1972). Previous studies of the formation of anthocyanin involved measurements of the total anthocyanin content of several pooled cotyledons and demonstrated the very precise control of the synthesis of anthocyanin by light and developmental state (Mohr, 1966; Drumm and Mohr, 1974; Steinitz et al., 1976).

Approaches such as those described above cannot answer the question of how the spatial pattern is established at the cellular level. Two changes of strategy are also necessary. A sensitive assay system is needed for screening responses of individual cells, accompanied by an analysis of the early stages of the response to light. In situ hybridization with homologous gene-specific probes for chalcone synthase (CHS) (Ehmann et al., 1991) satisfies both conditions. CHS catalyzes the first step in the synthesis of flavones, isoflavones, flavonols, anthocyanin, and related compounds (Hahlbrock and Scheel, 1989) and is a key enzyme at the beginning of the flavonoid-specific biosynthetic pathways.

Previous studies of the spatiotemporal pattern of the biosynthesis of anthocyanin led to far-ranging conclusions that were drawn exclusively from experiments in which entire cotyledons were stimulated. Stimulation, as well as analysis, must involve individual cells or individual clusters of cells. For this reason, we performed microbeam irradiation experiments and investigated the responses at the cellular level.

For more than a superficial understanding of photomorphogenesis, the following three questions need to be answered:

1. Can individual cells or cell clusters switch autonomously from dark to light development (Mohr, 1972)?
Figure 1. Spatial Distribution of CHS mRNA after Irradiation of the Entire Cotyledon.
Do cells coordinate their respective developmental programs via light-induced intercellular signals?

3. Is the spatial pattern genetically fixed, or does it develop in conjunction with information from the environment?

RESULTS

Spatial Distribution of CHS mRNA

The spatial distribution of CHS mRNA was assayed by in situ hybridization at the time when it is most abundant (48 hr after sowing); hybridization was performed with two gene-specific probes for CHS, SCHS1 and SCHS2, in series of parallel sections (Ehmann et al., 1991). Whereas no SCHS1-specific signal could be detected in the analysis of dark-grown plants (data not shown), the lower epidermis was strongly and uniformly labeled in plants grown in continuous far-red light, as shown in the left-hand panel of Figure 1A. CHS mRNA appeared to be less abundant in the upper epidermis (data not shown).

Continuous far-red light seemed to be less effective than continuous far-red light (Steinitz et al., 1976), and it caused a similar, patchy distribution of CHS mRNA in both the lower and the upper epidermis, with a generally weaker response in the upper epidermis (left-hand panel of Figure 1B).

To determine whether the pattern observed after irradiation with light was specific, the parallel section in each series was hybridized with SCHS2, a gene-specific probe for a second gene for CHS (Ehmann et al., 1991). Except for a somewhat decreased overall abundance of the corresponding CHS mRNA, the hybridization pattern (Figures 1C and 1D) was very similar to that obtained with SCHS1; this result was consistent with previous data (Ehmann et al., 1991). For each series, third and fourth parallel sections were treated with the respective sense strand probes (right-hand panel of Figures 1A to 1D). These controls were negative, suggesting that the observed binding of SCHS1 and SCHS2 to the epidermis is not due to a nonspecific affinity of mRNA for epidermal cells. The patchy, discontinuous distribution of CHS mRNA was not confined to one dimension but could be followed along the median axis through the entire series of parallel sections, as shown in Figures 1E to 1J. The spatial continuity of such labeled cell clusters through several parallel sections supports the hypothesis that the discontinuous pattern of the signal was due to the patchy distribution of CHS mRNA.

Spatial Distribution of Anthocyanin

For comparison with the spatial distribution of CHS mRNA, the spatial distribution of the final product, anthocyanin, was followed under the same conditions in the lower epidermis. Almost no pigment was present in the upper epidermis under these conditions (Mohr, 1966, 1972). In dark-grown plants, only a few cells contained anthocyanin and they were surrounded by nonresponding cells, as shown in Figure 2A. The overall response, as illustrated in Figure 3A, was weak with only faint staining in the tip of the leaf, along the margin, and along the veins. The strongest response (Figure 2C) was obtained after illumination with continuous far-red light, although many cells remained unpigmented or only faintly pigmented. Continuous red light caused an intermediate response, with staining extending from the tip, margin, and veins into the intercostal regions. This staining was discontinuous, with islets of well-stained cells distributed over a nonresponding background (Figure 2B).

Not All Cells That Express CHS mRNA Synthesize Anthocyanin

The attempt to correlate the spatial distribution of CHS mRNA with the pattern of staining for anthocyanin was only partially successful. Even after illumination by continuous far-red light, some unpigmented cells were interspersed among the well-stained cells (Figure 2C). When sections from such cotyledons were subjected to in situ hybridization, a homogeneous distribution of CHS mRNA was found throughout the lower epidermis (Figures 1A and 1C). Similarly, in cotyledons irradiated with continuous red light, the patchiness of the response was far more pronounced at the level of the end product anthocyanin than at the induction of CHS mRNA (Figures 1B, 1D, and 2B). Thus, tissues synthesizing anthocyanin were always found to contain CHS mRNA, but CHS mRNA was also present in many cells in which no anthocyanin was detected.

To follow the time course of the appearance of the discontinuous, patchy responses of individual cells (Figures 1C, 1D, and 2B), an induction experiment was performed using seedlings that had been grown for 42 hr in the dark. Under these conditions, the level of CHS transcripts was found to increase sharply within 30 min after a red-light pulse of 5 min duration that converted 87% of total phytochrome into the active state, as plotted in Figure 2D. A plateau was reached ~4 hr later.
Figure 2. Spatiotemporal Pattern of Formation of Anthocyanin after Irradiation of the Entire Cotyledon.
This induction was suppressed by a pulse of deep-red light (756 nm) administered immediately after the red-light pulse (Figure 2D). In the case of induction by a red-light pulse at 42 hr, strongly stained individual cells could be detected from 4 hr after the pulse at the tip of the cotyledon (Figure 2E). These cells developed into small anthocyanin-producing clusters located in the tip and along the margins and veins from 6 hr after induction (Figure 2F). From 8 hr after induction, the cluster increased in area and started to extend into the intercostal regions (Figure 2G). Figure 2H demonstrates that even 12 hr after induction, large clusters of unpigmented cells could frequently be observed and were still present a day later.

The Spatial Pattern of Anthocyanin Formation Can Be Manipulated by Microbeam Illumination

In microbeam illumination experiments, the selection of suitable regions of the cotyledon for irradiation is critical. Preliminary studies demonstrated the importance of the following four domains indicated in Figure 3A:

1. The tip of the leaf between the two lobes of the cotyledon, corresponding to an epidermal region of two to three rows of elongated cells: a region located ~10 cells to the left of center of the cotyledon was chosen for illumination throughout the experiments.
2. The marginal region of one to two rows of elongated cells: the microillumination was administered to the outermost part of the margin of the left lobe.
3. The cells adjacent to the vein, which are also elongated: the base of the first side vein that branches from the mid-vein into the left lobe of the cotyledon was selected for irradiation (at this location, the vein was three cells wide).
4. The cells of the intercostal lamina, which are small and isodiametric: the term "laminar stimulation" refers to microbeam illumination of these cells at the very center of the left lobe. Illumination of the elongated epidermal cells associated with the veins was carefully avoided.

The typical response of cotyledons to illumination over their entire surface by a pulse of red light was apparent as the synthesis of anthocyanin at the tip (data not shown), along the margin (Figure 3K), and along the veins (Figure 3I). This result was consistent with previous observations (Mohr, 1972; Steinitz and Bergfeld, 1977). However, 3 days after sowing, some anthocyanin was also seen in dark-grown cotyledons, predominantly at the tip, along the leaf margin, and along the veins (Figures 2A and 3A).

If the tip of the cotyledon was irradiated with a microbeam pulse of red light 2 days after sowing and the plants were then returned to darkness for another day, a localized increase of staining was observed in the region of the tip (Figure 3B). Similarly, if the microbeam was applied to the margin, anthocyanin was found predominantly around the site of irradiation (Figure 3C). Irradiation of the first side vein caused staining of the irradiated spot and the neighboring intercostal cells (Figure 3D). In addition, as the comparison of Figures 3K and 3L illustrates, the staining at the margin appeared to be suppressed. This finding corresponds to an inversion of the pattern observed after irradiation of the entire cotyledon.

Laminar stimulation induced a complex response. Frequently, we observed a halo of anthocyanin-producing cells that surrounded the site of irradiation, which itself was only faintly pigmented (Figure 3E). A strong response in the intercostal regions was usually accompanied by suppression of anthocyanin synthesis at the margin (Figure 3H). A comparison with the pattern obtained after illumination of the entire cotyledon (Figure 3I) showed even more conspicuous suppression of staining in the veins. The comparison with Figures 3F and 3J illustrates that, again, the pattern obtained after illumination of the entire cotyledon had been reversed. Rings composed of groups of stained cells often encircled a nonresponding core of ~10 to 20 cells in diameter, which, in the case of strong overall responses, resulted in cotyledons that resembled the photographic negative of a ladybug (Figure 3G).

Anthocyanin Synthesis Is Most Sensitive to Locally Perceived Fluences

The response to red light might be determined by the number of quanta absorbed, by the number of responding cells, or by a combination of both parameters. In an attempt to address this problem, the irradiated area was kept constant for each of the four irradiated sites, with only three cells being illuminated, and the fluence varied over three orders of magnitude. Alternatively, the fluence was fixed at 5.6 μmol m⁻² red light, as given by the vertical lines in Figures 4 to 6, and the illuminated area varied between 1 and 27 cells. The treatment...
Figure 3. Anthocyanin Patterns Induced by Microbeam Illumination in Various Leaf Domains.
required the identical handling of all cotyledons and even included focusing under a green safelight. As shown in Table 1, this extreme care was necessary because some of the responses were sensitive enough to be triggered to some extent by the green safelight, which is supposed to induce very little active phytochrome. The response was quantified independently for each of the four domains of the cotyledon.

Anthocyanin synthesis at the tip of the cotyledon was most efficiently induced by irradiation of the tip itself, as shown by Figure 4A. The response at the margin was induced best by stimulation of the margin (Figure 4B), the vein response by irradiation of the vein (Figure 4C), and the lamina response by stimulation of intercostal cells (Figure 4D). Thus, the response of each domain was strongest to irradiation of the same domain. This result suggests the localized induction of anthocyanin synthesis by light; this localization of induction is supported by the results of experiments in which the spot size was varied while the fluence was kept constant (Figures 4E to 4H). When neither the tip nor margin was irradiated, it was not possible to enhance the response in the irradiated domain by increasing the spot size (Figures 4E and 4F). By contrast, the responses of vein and intercostal cells after stimulation of the vein increased considerably when the light was distributed over a larger number of cells (Figures 4G and 4H). These results suggest strong cooperation among vein cells. In other words, the responses of vein and intercostal cells appear to be locally confined to a lesser extent than the responses of tip and margin cells.

Although for all four domains the locally perceived fluence was most efficient in inducing synthesis of anthocyanin (Figures 4A to 4D), the sensitivity toward this fluence varied between the domains of the cotyledon. In tip, margin, and vein cells, the response in darkness (Figures 4A to 4C) was relatively strong. Only a relatively small additional response could be evoked by local irradiation (Figures 4A to 4C). By contrast, in intercostal cells, in which an almost negligible dark response could be recognized (Figure 4D), a very strong additional response to light was observed (Figure 4D). Thus, dark responses and additional light responses appear to be inversely correlated.

Asymmetries in the Pattern of Anthocyanin Formation

For each individual seedling, asymmetries between the tip and the base or between the left (irradiated) and right (untreated) lobe of the cotyledon were recorded. Tip or vein irradiation often spread into the unirradiated lobes, obscuring any asymmetry conferred by the irradiation, as shown in Figure 5A. By contrast, irradiation of the leaf margin or laminar stimulation induced clear left-right asymmetry (Figures 5A and 5C).

Even dark-grown plants showed a high degree of tip-base asymmetry (Figures 5B and 5D), which was due to the strong dark response in the tip (Figure 3A). Irradiation of the tip increased this asymmetry to the maximum possible level (Figures 5B and 5D). Illumination of the vein reduced the frequency of tip dominance (Figures 5B and 5D). There were two reasons for this reduction: a second center of anthocyanin synthesis developed in the leaf base and its activity outweighed the tip responses, and an inhibition of anthocyanin formation at the tip was observed after microirradiation of the cells adjacent to the vein (Figure 4A).

Long-Range Suppression of Anthocyanin Formation

For each individual seedling, the staining at the margin and in the adjacent lamina was recorded and the response in the veins was compared to that in the surrounding intercostal cells. Margin suppression, wherein the margin stained less intensely than the lamina (Figures 3H and 3L), was found predominantly after laminar stimulation, as plotted in Figure 6A. Margin suppression was even more conspicuous after irradiation of the cells adjacent to the vein (Figure 6A). Some suppressive effect for large spot sizes was also achieved by tip irradiation (Figure 6C). The high sensitivity of the response to vein stimulation was demonstrated in a separate set of experiments, as shown in Table 1, in which treatment with the green safelight induced a partial suppression of anthocyanin synthesis. It should be emphasized that this suppression went lower than the dark level.
DISCUSSION

Can Individual Cells Switch Autonomously from Dark to Light Development?

Results of our microbeam illumination experiments showed clearly that the induction of anthocyanin synthesis by light is a local response (Figures 3 and 4). It is possible to induce a partial pattern and asymmetries (Figure 5). In other words, some parts of the cotyledon can switch from a dark- to a light-developmental program, whereas others maintain or enforce a commitment to dark development or skotomorphogenesis (as is especially conspicuous in the phenomenon of margin and vein suppression, see Figures 3F, 3H to 3L, and 6). The occurrence of spotted, patchy cell responses to nonsaturating stimuli (irradiation by continuous red light of the lower epidermis; see Figures 1C to 1D and 2B) during early phases of an inductive light response (Figures 2E to 2H) or in cells of low responsiveness (upper epidermis in Figures 1B and 1D) indicates that even individual cells can change from skotomorphogenesis to photomorphogenesis. Thus, the transition...

In dark-grown seedlings or in seedlings subjected to illumination of the whole cotyledon, the cells adjacent to the vein developed more anthocyanin than the surrounding intercostal cells (Figures 2A, 3A, and 3I). However, the converse of this situation was observed after laminar stimulation (Figure 6B), with large pigmented areas appearing in adjacent intercostal cells (Figure 6D). Such treatments resulted in a faintly tinted vein on a background of reddish intercostal cells (Figures 3F and 3J).
between the two developmental pathways appears to be cell autonomous.

What Might Be the Meaning of the Patchy Responses of Individual Cells?

The discontinuous, patchy responses of cells have a strong stochastic component. They were observed both in the case of the final product, anthocyanin, and in the case of its precursor activity, induction of CHS mRNA. The peculiar spatial distribution must, therefore, be due to some aspect of the earliest response to light. Such stochastic responses of individual cells are not restricted to phytochrome-induced phenomena but have also been found for the blue light–induced reorientation of cortical microtubules in the outer epidermis of phototropically stimulated maize coleoptiles (Nick et al., 1992). In this latter system, discontinuous, patchy responses of individual cells were also noted either during the early stages of the response to a saturating stimulus or as the final result of weak induction (Nick et al., 1992).

Patchy patterns therefore appear to mirror a general aspect of light-triggered signal transduction in plants. The high sensitivity of such responses requires a high degree of signal amplification during the first steps of the transduction chain, with resultant all-or-none outputs if responses are assayed at the level of individual cells. If all cells of a given organ were absolutely identical and homogeneous, even an extremely weak stimulation could result in the maximal response by the whole organ. It is clear that such a system would not have survived natural selection. It is the very heterogeneity of individual cells that is important and, thus, heterogeneity is not only tolerated by the plant, it is a clear necessity. Only in a heterogeneous population of cells can a transduction chain that involves extreme signal amplification lead to responses that gradually increase with increasing intensity of stimulation. Such gradual responses at the level of the whole organ are clearly necessary because the plant has to respond appropriately to stimuli that vary in intensity over several orders of magnitude.

Thus, the contradictory requirements posed by signal transduction in plants, that is, high sensitivity and a gradual response to varying levels of stimulation, are met by spatial separation: high sensitivity is achieved by signal amplification in individual cells, whereas the gradient of the response at the level of the whole organ is secured by interactions among members of a population of heterogeneous individual cells. The alternative to spatial separation, temporal separation, has been described by the term “sensory adaptation” (Galland, 1989): the receptors initiate from a highly sensitive state and undergo, upon saturating stimulation, a transition toward a less sensitive, adapted state that allows for more gradual responses.

### Table 1. Induction of Margin Suppression by Irradiation of the Vein with a Green Safelight Microbeam

<table>
<thead>
<tr>
<th>Response</th>
<th>Tip</th>
<th>Margin</th>
<th>Vein</th>
<th>Lamina</th>
<th>Tip–Base Asymmetry (%)</th>
<th>Margin Suppression (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment safelight</td>
<td>0.99</td>
<td>0.64</td>
<td>0.63</td>
<td>0.42</td>
<td>77</td>
<td>40</td>
<td>58</td>
</tr>
<tr>
<td>Control handling in the vein</td>
<td>0.84</td>
<td>0.32</td>
<td>0.61</td>
<td>0.50</td>
<td>61</td>
<td>60</td>
<td>82</td>
</tr>
<tr>
<td>Vein irradiation (5.6 μmol m⁻²)</td>
<td>0.82</td>
<td>0.09</td>
<td>0.88</td>
<td>0.70</td>
<td>41</td>
<td>95</td>
<td>65</td>
</tr>
</tbody>
</table>

Responses are given as the weighted averages of results from cotyledons, whose pigmentation was estimated for each domain, as described in Methods. Tip–base asymmetry is defined as the frequency of cotyledons with dominance of tip pigmentation over pigmentation at the base of the cotyledon. Margin suppression is quantified as the frequency of cotyledons with pigmentation in the margin, which was suppressed when compared to that of the cells in the neighboring intercostal lamina.
During positioning and focusing, seedlings were exposed to a monochromatic focusing safelight (0.07 μmol m⁻² sec⁻¹, Tₘₐₓ 69% at 550 nm, halfband width 17 nm). To estimate the effect of this focusing safelight, a further control group was treated identically but without the focusing treatment. However, except in the case of focusing on the vein (Table 1), no significant effect of the focusing could be detected (data not shown).

The red light stimulation was administered to the left lobe of the cotyledon with the right lobe serving as an internal control. The exact sites of these stimulations, referred to as tip, margin, vein, and laminary stimulations, are indicated in Figure 3A. After stimulation, which lasted for 1 min at most, the plants were returned to darkness for a further day until evaluation. Energy fluxes were measured with a radiophotometer (YSI model 65A; Yellow Springs Instrument Co., Yellow Springs, OH) with an additional dispersive, opaque shielding glass. Cotyledons were photographed under a stereomicroscope (Olympus) on Kodak Ektachrome slide film (400 ASA) at 60 times magnification.

Evaluation of Response

Formation of anthocyanin was monitored 1 day after induction because the response reached a final state 12 hr after induction (Figures 2E to 2H). For each of the four leaf domains (tip, margin, vein, and intercostal cells), the response was characterized by reference to one of five response classes: 0, no anthocyanin detectable; 1, staining of individual cells; 2, staining of leaf patches of 20 to 50 cells; 3, staining of large areas of 100 cells or more; 4, homogeneous staining of the entire domain. The occurrence of left–right or tip–basal asymmetry and suppression of margin and vein responses were also recorded.

The fluence–response data (Figures 4 to 6) represent the average scores from 70 individual seedlings for each data point; seedlings were collected from seven independent experiments performed on different days. For a fluence of 5.6 μmol m⁻² of red light, the diameter of the spot varied from 1 to 27 cells (Figures 4 to 6). In this experiment, we pooled the data from 35 individual plants for each data point with irradiation on two different days. The data presented in this study are based on results from more than 2500 microbeam illumination experiments. For each set of experiments, the total response (ΣEᵢpᵢ, with rᵢ being the response class of domain j of a given seedling i and pᵢ being the proportion of the total leaf covered by this domain) was compared to the values obtained from the very same cotyledons by a conventional spectrometric assay after hydrolytic extraction of anthocyanin (Brockmann et al., 1987). The coefficient of correlation between the values obtained by the two methods was 0.87.

ACKNOWLEDGMENTS

The work was supported by a grant from the Japanese Science and Technology Agency to P.N., financial support from the Frontier Research Program/Riken Institute to P.N., B.E., M.F., and E.S., a grant from the Sonderforschungsbereich (Grant No. 206, Signal-Transduction Chains) of the German Research Council to E.S. and B.E., a grant from the Landesgratuirtenforderungsprogramm to B.E., and financial support from the Human Frontier Science Program Organization to E.S. and M.F.

Received February 22, 1993; accepted March 30, 1993.

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